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Actinomycin D facilitates transition of AT domains in molecules of sequence (AT)nAGCT(AT)n to a DNAse I detectable alternating structure

Michael J.Lane*, Steven Laplante[1], Robert P.Rehfuss[1], Philip N.Borer[1] and Charles R.Cantor

Department of Genetics and Development, Columbia University College of Physicians and Surgeons, New York, NY 10032 and [1]Department of Chemistry, Syracuse University, Syracuse, NY 13210, USA

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ABSTRACT

The interaction of actinomycin D with (AT)nAGCT(AT)n (where n=2,3, or 4) was investigated using a combination of imino proton NMR and DNAse I digestion. The stoichiometry of the interaction appears to be one:one with the actinomycin chromophore intercalated between the two GC base pairs. This binding event facilitates the conversion of the flanking repetitive AT regions to an alternating conformation characterized by induced sensitivity of the ApT sequences to attack by DNAse I. The neighboring TpA sequences do not exhibit rate changes as a function of binding of the drug. The potential relevance of such ligand induced DNA structural alterations is discussed.

INTRODUCTION

DNA ligands can affect the structure of the DNA molecule to which they are bound. For example, an X-ray crystallographic study of Eco RI nuclease bound to its recognition sequence reveals radical kinks in the DNA caused by the bound protein (1,2). Similar changes in DNA structure at the site of ligand binding are consistently found in single crystal studies of antibiotics bound to short duplexes (reviewed in 3). DNA structural changes occurring at a distance from the principle interaction sites of bound ligands have been monitored by physical techniques such as electric dichroism (4,5), or inferred from equilibrium (6,7) and thermal melting studies (8,9). One example of such an inferred structural change is the stiffness of the linker region associated with the nucleosome core particle(10).

Other measurements consistent with such structural alterations away from ligand binding areas derive from footprinting experiments. Studies with both DNA bound drug

molecules and proteins have shown that regions distant from the binding sites can become more sensitive to endonucleolytic attack by DNAse I. Here we show that actinomycin D, a ligand which has been characterized extensively by many physical techniques (11-15), is capable of inducing a DNAse I detectable altered structure in d(ApT) sequences at a distance from its binding site. The pattern of altered DNase I cleavage is consistent with that observed by Klug and coworkers for long poly(dA-dT) sequences (16,17). The results suggest it may be feasible to monitor DNA mediated communication between two ligands bound to the same DNA molecule enzymatically.

MATERIALS AND METHODS

Oligonucleotide Synthesis and Purification Phosphoramidites for oligonucleotide synthesis were obtained from American Bionuclear. All other synthesis reagents were of HPLC grade and obtained from American Bionuclear, MCB, or Aldrich. Synthesis was carried out either by hand or using an Applied Biosystems synthesizer. The chemistry for such synthesis has been described by Tanaka and Letsinger (18). Base deprotection was carried out in concentrated ammonium hydroxide (Fisher) for no less than 18 hours at 55°C. Purification of oligonucleotides was done by electrophoresis through 20% polyacrylamide/7.0 M urea gels. Full length oligonucleotides were visualized by UV shadowing and eluted from the gel slice in 10mM Tris-HCl/1mM Na_2 EDTA, pH 8.0. Samples were stored frozen in distilled water after passage over a G10 sephadex column (Pharmacia) to desalt the material. Concentrations were determined spectrophotometrically at 260nm employing an extinction coefficient (assumed) of E(p)= 10,500 (19).

NMR Instrumentation and Sample Handling NMR spectra were obtained on a Bruker 360 MHz instrument. Samples consisted of 2.1 mM single strands in 10mM Tris-HCl/ 8mM $MgCl_2$/2mM $CaCl_2$, pH 7.5. in 10% D_2O. Water suppression was effected by the method of Hore (20). Carrier frequency was set at 3833 Hz downfield from water. Each spectrum is the result of 7000

scans. Assignment of imino protons was accomplished by studying the temperature dependence of the imino protons as described by Patel et al.(21). Actinomycin D was added as a solid to the oligonucleotide solution. Spectra were not recorded until at least 30 minutes subsequent to addition of the drug to ensure that the system was at equilibrium.

Oligonucleotide Handling End labeling was performed in 50mM Tris-HCl/10mM $MgCl_2$/1mM dithiothreitol (pH 8.9), using 20 units T4 polynucleotide kinase (BRL), 0.1 -1.0 ug oligonucleotide, and 0.1 mCi ^{32}P γ ATP (5000 Ci/mmol - Amersham) at 37°C for one hour. Labeled material was separated from unincorporated phosphate by electrophoresis on a 20% polyacrylamide/ 7.0 M urea gel. After visualization by autoradiography the oligonucleotide was eluted from the gel and precipitated from a 0.3 M sodium acetate/ 70% ethanol solution. Labeled sample was recovered in DNAse I buffer (10 mM Tris - HCl/ 8mM $MgCl_2$/2mM $CaCl_2$, pH 7.5) and the sample heated to 90°c and subsequently cooled to room temperature slowly (approximately 1°C per minute) to avoid snap - back of the strands with themselves. By native polyacrylamide gels the amount of such molecules in the preparations employed was not detectable.

DNAse I Digestions and Autoradiography DNAse I digestion parameters were rigorously controlled, for reasons we have previously detailed (22), such that 70+/-3% of the molecules remained uncleaved when the reactions were terminated. This generates a population of cleaved molecules greater than 80% of which have not been cut more than once. A typical reaction was performed in 8 ul as follows: 2 ul cold (carrier) ATATAGCTATAT, 2 ul labeled duplex, 2 ul actinomycin D (or buffer), and 2 ul DNAse I. Final AGCT site concentration was 10 uM in all reactions reported here. Enzyme was added last and the drug:DNA mixture was incubated for at least 30 minutes at 25°C prior to addition of enzyme to ensure that equilibrium was established. Digests were performed at 25°C and terminated after one minute by addition of 10 ul of a 10 M urea/ 100 mM NaOH/ 50 mM Na_2 EDTA/0.1% bromophenol blue/0.1% xylene cyanol solution.

Samples were then heated to 90°C for two minutes and quick chilled on ice prior to loading onto a 20% polyacrylamide/ 7.0 M urea sequencing gel. DNAse I patterns were visualized by autoradiography at -80°C using Kodak X-AR film for periods of up to one week. Densitometry was performed using a Joyce - Loebl Chromoscan 3. Intensifying screens were not used and band intensities were kept below an absorbance of 1.50 in order to ensure densitometric data was obtained in the linear response range of the film. All intensity values were determined as peak height.

Actinomycin D Handling Actinomycin D solutions were made fresh bimonthly and stored in the dark at 4°C. Concentrations were determined optically at 440 nm using a molar extinction coefficient of 24450 (6).

RESULTS

The electrophoretic pattern generated by the 5' end labeled, DNAse I singly cleaved, ATATAGCTATAT duplex in the absence and presence of varying total actinomycin D concentrations, is shown in Figure 1. At low total actinomycin to duplex ratios there is a notable enhancement of cleavage rate at the ATATAGCTATA*T phosphodiester bond. The enzyme cleavage rate at this position increases as the actinomycin D concentration is raised until the drug to duplex ratio reaches one:one. This is clearly visible in the densitometric scans of the autoradiogram shown in figure 2. The effect on the ATATAGCTATA*T bond is in contrast to the overall protection pattern produced by the ligand at other sites of cleavage. Furthermore, the extent of protection continues to change up to values exceeding two actinomycin D molecules added per dodecamer duplex. (We will address the stoichiometry reported by footprinting analysis more rigorously in a further communication - manuscript in preparation. However, the result suggests that DNAse I is competing with the actinomycin D for the DNA. This effect could explain previous footprinting experiments suggesting that two actinomycin D molecules were bound to the TATAGCGCTATA dodecamer (23).)

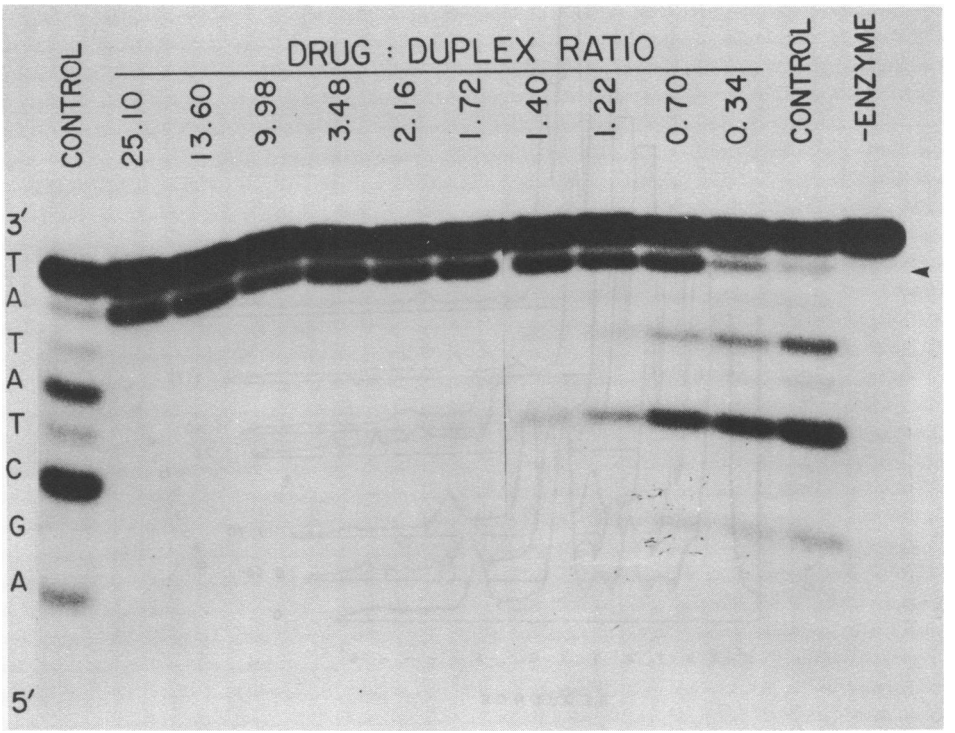

Figure 1. DNAse 1 digestion patterns of the duplex dodecamer d(ATATAGCTATAT) in the presence and absence of varying total actinomycin D concentrations. This autoradiogram is of digestion patterns analyzed by electrophoresis through a 20% polyacrylamide gel. Arrow indicates phosphate at which enhanced cleavage is noticed.

To rule out the possibility that the increase in enzyme rate at the ATATAGCTATA*T bond was due to binding of drug molecule to the end of the oligonucleotide duplex, we examined the imino protons in the presence and absence of actinomycin D by NMR. Assignment of ATATAGCTATAT imino proton peaks was carried out by studying the duplex at various temperatures. Peak assignments are indicated in Figure 3a along with appropriate spectra. Assignment of imino protons in the drug bound and drug free states was aided by the observation that only the GC imino proton peaks shifted substantially in the presence of actinomycin D. The

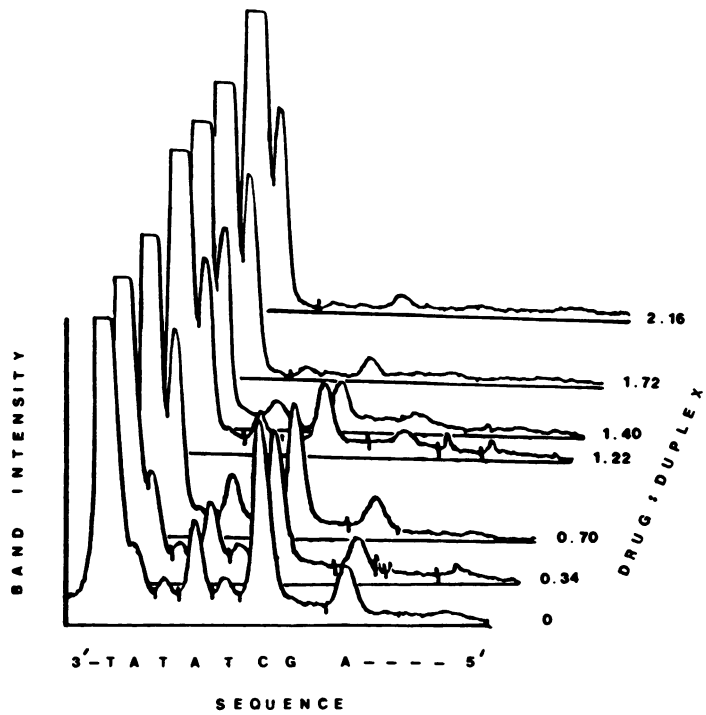

Figure 2. Densitometric scans of selected lanes from autoradiogram shown in Figure 1. Axes are labeled. Enhanced cleavage is noticeable at only the ApT phosphate closest to the 3' end of the duplex.

spectra in Figure 3b show the behavior of the imino protons at varying total actinomycin D:duplex dodecamer ratios. Broadening of the visible AT imino protons presumably arises from the slight asymmetry of the bound drug molecule which contains a pseudo two-fold symmetry axis. The relative lack of perturbation of the AT imino protons seems somewhat surprising in light of the work of Patel and coworkers (24,25) with actinomycin D and the dodecamer CGCGAATTCGCG. It seems likely that the presence of two actinomycin D binding sites on the CGCGAATTCGCG duplex leads to greater perturbation of the AT protons in that compound. We conclude from our own measurements on ATATAGCTATAT that the actinomycin molecule is intercalated in the GC site at low molar drug to duplex dodecamer ratios. This conclusion is

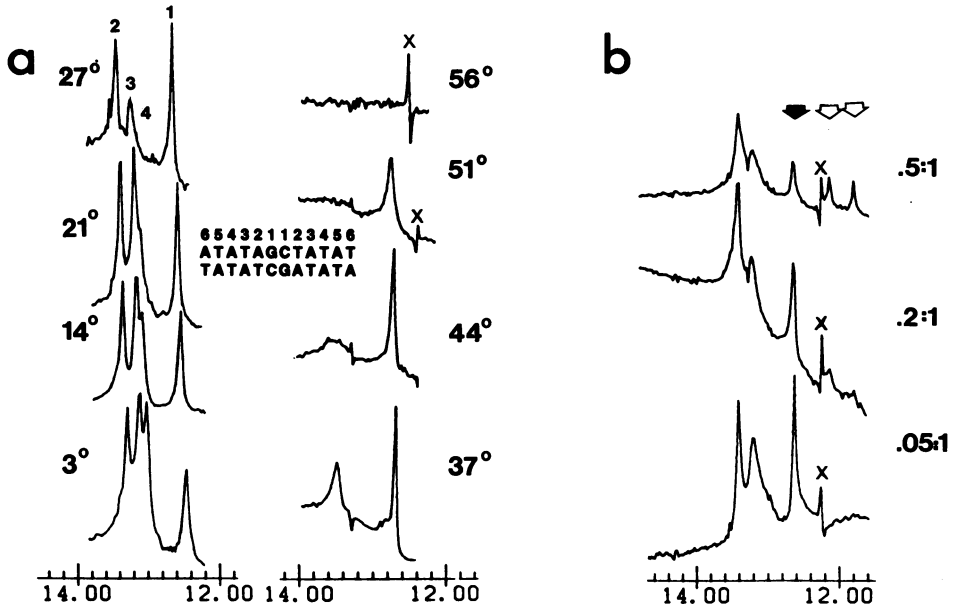

Figure 3. Proton NMR spectra of the imino protons of the dodecamer duplex ATATAGCTATAT. a) Temperature dependence of the imino protons of the duplex. Proton number assignments are shown in the inset and corresponding assignments are indicated over the 27°C spectra. b) Spectra generated at less than stoichiometric concentrations of actinomycin D. Filled arrow indicates unbound GC imino proton, unfilled arrows the upfield shifted and split bound GC imino protons.

consistent with NOE measurements on the actinomycin D : AGCT tetranucleotide complex (26).

The enhancement in DNAse I cleavage rate at the most terminal phosphate in the dodecamer implies a fairly long range effect extending a distance of five base pairs from the intercalation site. However, an alternative explanation would be a transition in structure occurring continuously between the actinomycin D intercalation site and the end of the dodecamer of a sort not detectable by our NMR probing of the imino protons. If one assumes that the duplex is B form DNA, the enzymatically hypersensitive phosphate is closest in space to the 5'T*AGCT 3' phosphate moiety on the opposite strand. This suggests that if one could examine the phosphates closer to the 5' end of the duplex it might be

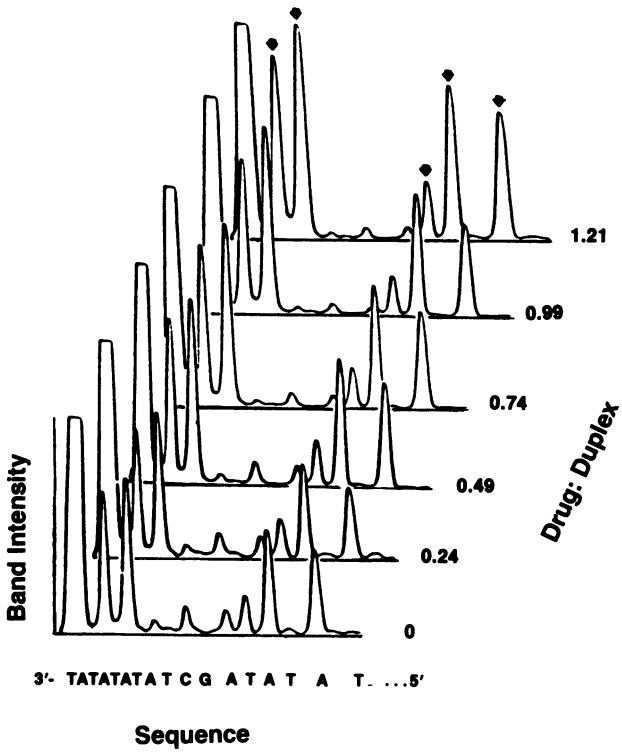

Figure 4. Densitometric scans of DNAase I partial digests of the ATATATATAGCTATATATAT end labelled 20-mer duplex in the presence and absence of varying total actinomycin D. As the drug is added the cleavage frequency at ApT bonds increases. Total drug to duplex was kept low to avoid potential non-specific binding of actinomycin D to the AT domains. Bonds which show enhanced cleavage are indicated by arrows. Note enhanced cleavage at ApT bonds relative to both the AGCT domain and the TpA bonds in the absence of actinomycin D relative to the pattern displayed in the absence of drug in figure 2.

possible to observe structural alterations closer to the bound ligand. Such an experiment is hampered in the dodecamer by the requirement of the nuclease for an opposing phosphate on the other strand of a DNA molecule to effect cleavage at a given phosphodiester bond (27). The last four phosphodiester bonds at the 5' end are thus inaccessible to the enzyme in the dodecamer. To examine this question we

increased the total duplex length to twenty by using the molecule ATATATATAGCTATATATAT. This compound maintains the purine - pyrimidine base stacking motif of the dodecamer. In this 20 - mer the DNA strands are long enough to present to the enzyme an opposing phosphate at the base steps between the drug intercalation site and the enzymatically sensitive phosphate found on the dodecamer, albeit on the other strand of the duplex.

Densitometric scans of DNAse I cleavage products of the ATATATATAGCTATATATAT molecule in the presence and absence of actinomycin D are shown in figure 4. It is apparent that the rate of cleavage of each of the dApT phosphodiester bonds observable becomes greater as actinomycin D is titrated into the system, relative to their respective cleavage rates in the absence of the ligand although to a lesser degree than in the dodecamer. An enhanced cleavage rate is also visible at the unique T*AGC phosphodiester bond. A key to what is happening in the AT domains is suggested from a comparison of the enzymatic cleavage rates of the two molecules in the absence of actinomycin D. Comparison of the minus drug DNAse I digestion patterns of the two molecules, in figures 1 and 3, shows that increasing the length of the alternating AT domain favors cleavage at the ApT phosphodiester bonds relative to both the TpA phosphodiester bonds and the central AGCT domain. This is similar to what is observed when the DNAse I cleavage patterns of poly(dA-dT) and the ATAT tetramer are compared (16,17) leading us to believe that the binding of actinomycin D to the central portion of these oligonucleotide duplexes somehow alters the equilibrium of the AT domains in such a way as to favor an alternating enzyme sensitivity. It is tempting to suggest that this is a consequence of reshuffling of the flanking base stacks caused by intercalation of the actinomycin chromophore. While the enhanced cleavage rates are consistent with the alternating B DNA helix suggested by the crystal structure of d(AT)$_2$ (28), Assa-Munt and Kearns (29) found no evidence for an alternating structure involving the sugar-phosphate backbone in poly(dA-dT) by 2D NMR methodology. It remains

TABLE 1. DNAse I cleavage ratios for (AT)nAGCT(AT)n in the absence and presence of actinomycin D.*

MOLECULE	ApT / TpA	ApT(+drug)[a] / ApT(-drug)	TpA(+drug)[a] / TpA(-drug)
$AT_2AGCTAT_2$	3-4	3-4	—
$AT_3AGCTAT_3$	12-15	1.8-2.1	1[b]
$AT_4AGCTAT_4$	>40	1.3-1.5	1[b]

*) All band intensities were determined densitometrically as peak height.

a) Ratios of bands in the presence of 1.2 equivalents of actinomycin D per AGCT duplex site to the same bands in the absence of drug.

b) Values for these base steps showed no systematic variation as a function of actinomycin D concentration.

possible that a more subtle alternating configuration could be the cause of the observed phosphodiester preference largely based on the presence of two peaks in the ^{31}P NMR spectrum of poly(dA-dT)(30).

Finally, if our interpretation of the 12-mer and 20-mer digestion experiments were correct then we would predict that an oligonucleotide of sequence ATATATAGCTATATAT (16-mer) would show both intermediate values of drug induced ApT enhancement (e.g. a larger drug induced rate enhancement than shown in figure 4 but less than observed in the dodecamer) and an intermediate ApT/TpA cleavage ratio. This is exactly what we observed when we subsequently synthesized and digested this molecule(experiments not shown). Table 1 summarizes the relevant cleavage ratios for the three oligonucleotides. We note that the relative invariance of the TpA ratio in the presence and absence of the drug implies that intercalation of actinomycin into the GC position changes only the ApT and not the TpA base stacking geometry. An important additional conclusion to be drawn from this invariance is that we are not observing a kinetic displacement effect.

The enzyme rate data are summarized in figure 5 from two different perspective drawings of the 20-mer helix. In 5a the base-pairs are shown perpendicular to the helix axis and the position of the drug inferred from the enzyme digests and NMR experiments is indicated. From this perspective the

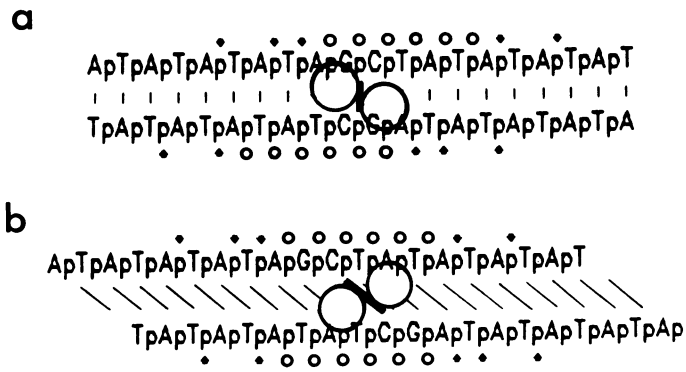

Figure 5. Summary of changes in DNAse I digestion kinetics on (AT)nAGCT(AT)n duplexes caused by the binding of actinomycin D. In a, the helical perspective is the classical one - with the base pairs perpendicular to the helix axis. This perspective shows the binding of the drug (inhibition of cleavage - circles) as skewed toward the 3' end of the duplex. Arrows indicate those phosphodiester bonds which show drug induced enhanced cleavage rates. In b, the perspective suggested by Drew (23) based on the phosphate recognition properties of the DNAse I enzyme is shown. Here it is evident that the asymmetry is due to enzymatic recognition properties not asymmetric drug binding.

footprint produced by the ligand appears to be larger than expected from the size of the drug molecule. In contrast figure 5b represents the duplex as it is thought to be recognized by the enzyme molecule (26). This representation illustrates that enzyme inhibition is due to simple masking of the phosphates surrounding the drug site. Note that preventing the enzyme from contacting a single phosphate will inhibit cleavage at the opposite phosphate across the minor groove. We feel this perspective better illustrates the DNAse I cleavage patterns, both drug induced and uninduced.

DISCUSSION

Recent studies have shown that regions of DNA surrounding eukaryotic and prokaryotic genes are capable of adopting altered conformations under the influence of torsional strain provided by supercoiling. Regions such as poly(GA)n:poly(CT)n, capable of adopting altered configurations under such tension, are also capable of

maintaining an altered structure in linear DNA depending on length and the influence of B-type flanking sequences(31). These same sequences are hypersensitive to nuclease attack in actively transcribing genes in eukaryotic cells, suggesting that such genes may be under torsional strain *in vivo* (32-34). Recent evidence has been presented suggesting that such altered sequences can influence flanking, putative B DNA when under the tension provided by supercoiling(35). The data presented here allow an alternative hypothesis. Conformationally *adaptive* sequences may exist adjacent to protein recognition sites which can undergo a less dramatic, enzymatically detectable transition, induced by the binding of ligand at a distance from the ligand interaction site. This concept and experiments to justify it have been reviewed recently(36). Drew and Travers (37) have recently pointed out that deproteinized nucleosomal DNA appears to have a defined sequence complexity as determined by statistical footprinting. Their findings and the earlier results of Keene and Elgin (38) suggest that evolutionary selection for DNA sequences capable of deforming to accommodate ligand binding, as well as coding sequence, may occur. Such sequence adaptivity need not be obvious at the level of rudimentary primary sequence comparison.

The experimental approach described here: using a small defined DNA sequence and a small ligand to perturb DNA structure, may be helpful in evaluating the structural potential (adaptivity) of DNA sequences systematically. Methods for DNA synthesis are now capable of producing large amounts of short molecules; thus techniques such as Raman spectroscopy and NMR can be used to further characterize sequences which appear to show adaptive behavior in nuclease studies.

As this manuscript was approaching completion, Suggs and Wagner described stable maintenance of the induced structure we describe here in an AT_{14} repeat, ligated to flanking putative B stretches of DNA, detectable by five independent probes(39). Their report lends support to our structural interpretation of DNAse I cleavage rates.

*Present address: SUNY Health Science Center, Syracuse, NY 13210, USA

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